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Androgen-dependent stimulation of brain dopaminergic systems in the female European eel (*Anguilla anguilla*)

Short title: Androgen stimulation of brain dopamine systems

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Abstract

Dopamine, a neurotransmitter present in all vertebrates, is involved in processes such as motor function, learning and behavior, sensory activities, and neuroendocrine control of pituitary hormone release. In the female eel, we have analyzed how gonadal steroids regulate brain expression of tyrosine hydroxylase (TH), the rate-limiting enzyme in the biosynthesis of dopamine. TH mRNA levels were assayed by quantitative real-time RT-PCR. TH-positive nuclei were further localized by in situ hybridization (ISH) and immunohistochemistry, and the location of TH nuclei that project to the pituitary was determined using DiI retrograde tracing. Chronic in vivo treatment with testosterone increased TH mRNA specifically in the periglomerular area of the olfactory bulbs, and in the nucleus preopticus anteroventralis (NPOav). NPOav was DiI-labeled showing that this nucleus is hypophysiotropic in the eel. The non-aromatizable 5□-dihydrotestosterone gave identical results in both areas, whereas estradiol-17□ had no stimulatory effect, showing that the observed stimulatory effects of testosterone were androgen-dependent. In teleosts, dopamine neurons originating from the NPOav directly inhibit gonadotropic function, and our results indicate an androgen-dependent positive feedback on this neuroendocrine control in the eel. In mammals, dopamine interneurons in the olfactory bulbs are involved in the enhancement of olfactory sensitivity and discrimination. Our results in the European eel suggest an androgen-dependent stimulation of olfactory processing, a sensory function believed to be important in eel navigation during its reproductive migration towards the oceanic spawning grounds. To our knowledge, this is the first evidence from any vertebrate of an androgen-dependent effect on dopaminergic activity in the olfactory bulbs, providing a new basis for understanding the regulation by gonadal steroids of central dopaminergic systems in vertebrates.

Introduction

Dopamine (DA) is a vertebrate neurotransmitter essential in processes such as motor function, learning and behavior, and sensory activity, e.g. olfactory processing (1, 2). Neuroendocrine control of pituitary hormone release is also among the versatile functions of DA. In the mammalian brain, hypothalamic factors are released into the median eminence and transported to the anterior pituitary via the hypothalamo-pituitary portal system. Teleost fishes lack the median eminence and instead have a direct innervation of the adenohypophysis (3). Despite this difference in the neuroanatomical organization, DA has been shown to exert similar control on the release of pituitary hormones, such as prolactin (4, 5), thyrotropin (6, 7), growth hormone (8, 9), ACTH and □-melanocyte-stimulating hormone (10, 11), in mammals and teleosts.

Among teleosts, the functionally best-characterized DA system is the neuroendocrine system responsible for the inhibitory control of gonadotrope activity (12, 13). In some teleosts, this system inhibits the final stages of gametogenesis through DA neurons originating from the antero-ventral preoptic region (nucleus preopticus anteroventralis; NPOav) and directly innervating the pituitary gonadotropes (14). This DA system has been shown in adult teleosts to be regulated by estradiol-17□ (E2). E2 seems to increase the DAergic tone, thereby exerting

an inhibitory control on the ovulatory surge in LH release. Indeed, estrogens are considered key-steroid effectors in the brain of lower vertebrates, available to the CNS either through synthesis in the gonads and transport by the circulatory system, or via local aromatization of androgens (15-17). Androgen-dependent effects on DA activity have rarely been investigated, and such effects have not previously been reported in teleosts.

The European eel, *Anguilla anguilla* (order Elopomorpha), provides a relevant model to study the functionality of brain DA systems in vertebrates. First, as a teleost, the eel has a reduced brain noradrenergic system, with cell bodies confined to the hindbrain (18). Second, as a member of the elopomorphs, an early branching teleost order, the eel may have conserved characteristics that are less derived compared to most other teleost groups. Thus, eels could provide information on ancestral regulations of vertebrate DA systems (19). Moreover, because of its peculiar life cycle, the female European eel provides a powerful model for experimental investigations on the effects of gonadal steroids on the CNS (e.g. 20). If prevented from its oceanic migration towards its spawning grounds in the Sargasso Sea, puberty is completely arrested in the eel (21), and we recently showed that DA is responsible for this prepubertal block of gonadal development (22). However, which factors set up and regulate this DA inhibition of puberty, remains unknown. Prior to its reproductive migration, the eel undergoes several physiological and morphological changes, including growth and differentiation of the olfactory system, which is believed to be crucial for navigation during migration and spawning (23). In addition, there is an increase in plasma steroid levels, which in teleosts include both estrogens and androgens (24, 25). Considering the relatively high androgen plasma levels in female eels, and the fact that the eel has an unusually low brain aromatase activity compared to other teleost species (26, 27), it was of interest to investigate how androgens regulate central DA systems in the female prepubertal eel.

Tyrosine hydroxylase (TH) is the rate-limiting enzyme in the biosynthesis of catecholamines (28). As essentially all catecholaminergic neurons in the teleost fore- and midbrain are DAergic (18), quantification of TH mRNA reflects central DAergic activity (29). In this work, we have investigated the regional brain expression of TH and its regulation by gonadal steroids using quantitative real-time (qrt)RT-PCR. TH in situ hybridization and immunohistochemistry were used to further characterize the distribution of specific TH brain nuclei regulated by steroids, and the location of TH nuclei that project to the pituitary was determined using DiI retrograde tracing. To the best of our knowledge, these results provide the first evidence of an androgen-dependent regulation of dopaminergic activity in the olfactory bulbs in a vertebrate. Some preliminary data were presented in a short note (30).

Materials and Methods

Animals

Female European eels (*Anguilla anguilla* L.) were netted in the Loire River in western France (November 2003) during their downstream migration, which represents the start of their reproductive migration towards the Sargasso Sea. Downstream migrating eels (also called “silver eels”) are all at a prepubertal stage (31). The eels were transported to the laboratory at MNHN (Paris, France), and kept under a natural photoperiod in running aerated freshwater at about 15 °C (3 - 4 eels / 100 l tank). Because eels undergo a natural starvation period at the silver stage, they were not fed. Animal manipulations were performed under the supervision of authorized investigators (C.P., S.D.) according to French regulations and the European Convention on Animal experimentation for scientific research.

In vivo steroid treatment

Eels received weekly perivisceral injections of 2 mg steroid (suspended in saline) / kg body weight (BW). This injection protocol with a minimal duration of 6-8 weeks gives a stable and physiologically relevant plasma concentration of the injected steroid (e.g. plasma testosterone, T, was15 ng/ml after injections in the T-treated eels, compared to < 1 ng/ml in the control). This protocol has been previously used to investigate steroid feedback mechanisms on various brain and pituitary targets in the eel (e.g. 20, Huang et al, Endocrinology, 1999).

Experiment 1: The effect of T or E2 on the amount of TH transcripts

Twenty-four female eels with an average BW of 298 ± 112 g were randomly distributed into three experimental groups (n = 8 eels/group). The eels received weekly injections for 8 weeks of T (Sigma, St Louis, MO, USA), or estradiol-17□ (E2, Sigma), or of vehicle alone (control eels). All sampled brains were used for quantification of TH transcripts by qrtRT-PCR (see below).

Experiment 2: The effect of T, DHT, or E2 on the amount of TH transcripts

Forty-eight female eels with an average BW of 344 ± 57 g were randomly distributed into four experimental groups (n = 12 eels/group). The eels received weekly injections for 8 weeks of either T, E2, 5□-dihydrotestosterone (DHT; a non-aromatizable androgen, Sigma), or vehicle (control). Brains from eight eels per group were used for TH transcript analyses by qrtRT-PCR, while the remaining brains from each group were fixed and used for TH in situ hybridization analyses (see below).

Sampling procedure

At the end of each experiment, eels were killed by severing the medulla oblongata. For TH transcript analyses by qrtRT-PCR, brains were removed quickly, dissected into six different parts ((a) olfactory bulbs, (b) telencephalon including the rostral preoptic area, (c) optic lobes, (d) di-/mesencephalic areas, (e) cerebellum, and (f) medulla oblongata) (Fig. 1) and stored in 0.5 ml RNAlater™ (Ambion, UK) at –20 °C until further processing. Ovaries and liver were dissected out and weighed for calculation of gonado-somatic index (GSI, [ovary weight/BW] x 100) and hepato-somatic index (HSI, [liver weight/BW] x 100). Steroid treatments did not induce any significant change in GSI nor HSI (Table 1).

Quantitative real-time RT-PCR of eel TH

RNA extraction and cDNA synthesis

Brain parts (5 - 50 mg) were homogenized using a FastPrep instrument (Qbiogene, CA, USA) and total RNA was extracted using the FastRNA Pro Green kit (Qbiogene). Following DNase I treatment (DNase Free™, Ambion, UK), all samples were ethanol-precipitated to remove traces of salts, enzymes, and other possibly interfering substances. First strand cDNA was synthesized in 50 µl reactions with 2 µg total RNA as template. The RNA was pre-incubated with 2 µg random hexamer primers (Promega, France), before the RT reaction was set up using 100 nmol dNTPs, 56 U RNase inhibitor, and 400 U Moloney murine leukemia virus reverse transcriptase (MMLV-RT) (all Promega).

Primers and reference gene

We used acidic ribosomal phosphoprotein P0 (ARP) as reference gene in the qrtRT-PCR because 1) brain transcript levels of this gene are similar to those of TH, thus increasing the validity of the calculated relative expression levels, 2) the presence of introns in the gene permits the design of primers covering exon-exon borders to avoid amplification of potential traces of genomic DNA, and 3) the ARP mRNA expression does not vary with experimental treatments or developmental stage (32). Gene-specific primer design was based on the mRNA sequences of European eel TH (GenBank accession number AJ000731; 33) and European eel ARP (GenBank accession number AY763793; 32): THfw GCC CAG TTT TCT CAG AAC ATT G, THrv TGC ACC AGC TCT CCA TAG G (TH amplicon size 170 bp), and ARPfw GTG CCA GCT CAG AAC ACT G, ARPrv ACA TCG CTC AAG ACT TCA ATG G (ARP amplicon size 107 bp). Primers were designed with the Primer3 software (Whitehead Institute/MIT, USA) and purchased from MWG-Biotech AG (Germany). All primers had T_ms between 58 – 60 °C, and a GC content between 42 – 58 %, avoiding more than three consecutive Gs. One primer in each pair was designed in a cDNA exon-exon border.

Cyber-green assay

The assay for eel TH was set up using the Light Cycler system with SYBR Green I sequence non-specific detection (Roche Diagnostics). After an initial Taq activation at 95 °C for 10 min, 42 cycles of PCR were performed using the LightCycler with the following cycling conditions: 95 °C for 15 s, 60 °C for 5 s, 72 °C for 10 s, and a fourth segment of 83 °C for 5 s. Each PCR reaction was performed in a total volume of 15 □l, made from diluted cDNA template (4 □l), forward and reverse primers (7.5 pmol each), and SYBR Green I master mix (3 □l). See (32) for further details.

Light Cycler PCRs for target and reference genes were always run in duplicate from the same cDNA dilution taken from the same RT reaction. For each brain part, serial dilutions of a cDNA pool made from several samples were set up and run in triplicate both of target gene and reference genes to assess PCR efficiency and which dilutions to use for the unknown samples. Calculation of PCR efficiency (E) was based on the slope of the relationship between log input cDNA vs. the threshold cycle (C_t, defined as the point where the fluorescence increases above a background threshold level, which in our case is determined as the second derivative maximum): $E = 10^{-(1/slope)}$. Each assay (32 capillaries) included a calibrator (which is an arbitrarily selected sample necessary to adjust for assay to assay variations), consisting of whole brain cDNA material, run in duplicate for both target and reference genes. The unknown samples were expressed as the fold difference from the calibrator. As quantification of relative expression levels with the $\Delta\Delta C_t$ method was not possible (i.e. PCR efficiency lower than 100 %), we used an efficiency-corrected relative expression method (34):

$$\text{Relative expression} = E_{target}^{\Delta Ct\,(calibrator - sample)} * E_{reference}^{\Delta Ct\,(sample - calibrator)}$$

Each assay included no-template-controls (substituting cDNA with water) for each primer pair to confirm that reagents were not contaminated. Substituting cDNA with RNA in the PCR reactions verified the absence of interfering residual genomic DNA. The assays were only used within the C_t range where there was a linear relationship between log input cDNA vs. C_t for the serially diluted cDNA pool. Melting curve analysis, gel electrophoresis, and sequencing assessed the identity of the products.

Brain histological procedures

Tissue preparation

For all histological preparations, eels were anaesthetized by immersion in MS 222 (Sigma) and perfused through the aortic bulb with 0.65 % NaCl in 0.1 M phosphate buffer (pH 7.4) followed by 4 % paraformaldehyde in phosphate buffer. Upon fixation, the skull was removed and the brain with pituitary attached was carefully dissected out and stored overnight in fresh fixative at 4 °C.

For the in situ hybridization (ISH) and immunohistochemical analyses, the brains were rinsed in phosphate buffered saline (PBS, pH 7.4), and immersed overnight at 4 °C in a cryo-protective solution of PBS with 15 % sucrose. Subsequently, the fixed tissue was placed in inclusion moulds, embedded in Tissue-Tek (Miles, USA), frozen in cold isopentane, and stored at –80 °C.

The neuroanatomical terminology used for description of brain nuclei is based on the nomenclature of the brain atlas of Japanese eel (35).

TH in situ hybridization (ISH)

To specifically localize the TH-expressing nuclei in the eel brain, and furthermore as support for the qrtRT-PCR expression analyses in the different brain regions, in situ hybridization (ISH) analysis was performed on control female eels. To further clarify which specific brain nuclei are up- or down-regulated by sex steroid treatment, ISH was also performed on females from each of the steroid-treated groups (T, DHT, E2). cRNA probes were produced from eel TH cDNA (33). Eel TH inserted into pGEM-T easy transcription vector (Promega) was linearized and transcribed using Sp6/T7 RNA polymerase from the corresponding promoter. The cRNA probes were labeled by insertion of digoxigenin-UTP nucleotides according to the manufacturer's protocol (Roche,

France), using 1 □g linearized cDNA as template. The length of the cRNA probes was checked by electrophoresis. Sense probes transcribed from the opposite promoter were used as negative controls.

The ISH procedure was carried out on 20 □m transversal sections cut with a cryostat (Leica, Germany), and thaw-mounted on Superfrost plus slides (Gassalem, France). The specific staining procedure is described elsewhere (36). Stained sections were analyzed and digitally photographed under a Leica DMRB microscope.

TH immunohistochemistry

To further elucidate the brain distribution of TH neurons, especially focusing on hypophysiotropic fibers, immunohistochemistry was performed using control female eels.

The immunohistochemical procedure was carried out on 15 □m transversal sections cut with a cryostat (Leica, Germany), and thaw-mounted on Superfrost plus slides (Gassalem, France). Sections were pre-incubated for 1 h in 1 % normal sheep serum (Sigma) in PBS and 0.1 % Triton. The sections were subsequently incubated overnight at room temperature in a 1:500 dilution of rabbit anti-rat TH polyclonal antisera (Institut Jacques Boy, France) (Thibault J, et al ; 1981) in 1 % normal sheep serum and 0.1 % Triton. This antiserum has been used successfully to reveal TH-positive neurons in trout (59). After rinsing several times in PBS, sections were exposed to peroxidase-conjugated goat anti-rabbit Fab fragments (1:100, Biosys, France) for 1 h. The peroxidase activity was visualized as a blue product using 0.3 % 4-chloro-1-naphtol and 0.03 % hydrogen peroxide (Sigma). The sections were mounted in PBS/glycerol and cover slipped. Stained sections were analyzed and photographed under a Leica DMRB microscope.

Retrograde tracing from the anterior pituitary

To further characterize hypophysiotropic brain nuclei in the eel, a retrograde tracing was performed according to (37) on female eels (BW 200 - 300 g). Upon fixation, the ventrorostral surface of the pituitaries was dried using filter paper, before a small hole was made along the midline of the pars distalis using an insect pin. A microcrystal of DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindicarbocyanine perchlorate) was subsequently implanted with the insect pin (Fig. 2). Brains with attached pituitary were then submerged in 4 % paraformaldehyde in 0.1 M phosphate buffer and incubated in darkness at 37 °C for 2 - 8 weeks. The brain/pituitaries were then embedded in 10 % gelatin in phosphate buffer and transversally sectioned at 50 □m with a vibratome. The sections were mounted in glycerol: phosphate buffer (3:1), and analyzed under a Leitz fluorescence photomicroscope equipped with rhodamine filters.

Data analysis

Data are presented as mean ± standard error of mean (SEM). Statistical analyses were performed using InStat 3.0b (GraphPad Software, CA, USA). Means of relative TH mRNA levels in different brain regions and between treatment groups were compared by the Kruskal-Wallis test (non-parametric ANOVA) followed by Dunn's multiple comparisons post test. The level of significance was set at 0.05.

Results

Brain distribution of TH mRNA as measured by qrtRT-PCR

There was a distinct spatial distribution of TH mRNA expression in female prepubertal eels as analyzed by qrtRT-PCR (Fig. 3): Highest relative levels were found in the olfactory bulbs, where the expression was about 10 - 20 times higher than in the diencephalic / mesencephalic areas and the telencephalon / rostral preoptic area. TH was detectable at low levels in the optic tectum (including dorsal tegmentum) and medulla oblongata, while no TH expression could be detected in the corpus cerebellum or the pituitary. In situ hybridization (ISH) and immunohistochemical analyses were in agreement with the neuroanatomical distribution of TH mRNA as revealed by qrtRT-PCR.

Effect of steroid treatment on TH mRNA expression as measured by qrtRT-PCR

Chronic in vivo steroid treatment affected TH mRNA levels in female prepubertal eels. In Experiment 1, we compared the effect of E2 and T on the amount of TH transcripts (Fig. 4). T-treatment significantly increased TH mRNA levels in the olfactory bulbs (4.7-fold; $P < 0.05$) and telencephalon / rostral preoptic area (3.2-fold; $P < 0.01$). No significant effect of T was seen in the optic tectum, diencephalic / mesencephalic areas, or medulla oblongata. Treatment with E2 on the other hand, reduced TH mRNA levels specifically in the diencephalic / mesencephalic areas (E2 vs. T: 3.7-fold reduction, $P < 0.01$; E2 vs. control: 2.6-fold reduction, *NS*). No effect of E2 was found in other brain regions, including the olfactory bulbs and telencephalon / rostral preoptic area.

In order to test the specificity of androgenic versus estrogenic effects on the expression of TH, we performed a second experiment (Experiment 2), treating female silver eels with T, DHT (a non-aromatizable androgen), or E2. As in Experiment 1, T-treatment increased TH mRNA levels relative to control specifically in the olfactory bulbs (2.3-fold; $P < 0.01$) and telencephalon / rostral preoptic areas (2.5-fold; $P < 0.01$) (Fig. 5). There was no effect of T treatment in the other regions. Similar to T, treatment with DHT specifically increased TH mRNA levels in the olfactory bulbs (2.3-fold; $P < 0.01$) and in the telencephalon / rostral preoptic area (2.5-fold; $P < 0.01$), with no effect in the other regions. E2-treatment, on the other hand, affected TH transcript levels in the diencephalic / mesencephalic areas, resulting in a 2-fold reduction compared to control ($P < 0.05$). The E2 group was similar to control in all other brain regions, including the olfactory bulbs and telencephalon / rostral preoptic areas (Fig. 5). The fact that treatment with T and DHT gave identical results shows that the stimulatory effect of T on TH mRNA levels in the olfactory bulbs and telencephalon / rostral preoptic areas is androgen-dependent.

Localization of TH neurons by in situ hybridization – effects of androgen treatment

To further localize the effects of androgens as demonstrated by the qrtRT-PCR analysis, we investigated the cellular distribution of TH transcripts by ISH in brains taken from controls and steroid-treated eels. In the olfactory bulbs, TH mRNA-expressing cells were found throughout the periglomerular layer (Fig. 6A). In androgen (T or

DHT) -treated eels, ISH analyses showed a considerably higher TH mRNA labeling in all parts of this bulbar layer compared to control eels (Fig. 6B). No difference in TH ISH labeling was observed after E2 treatment (data not shown). This is in accordance with the results from the qrtRT-PCR analyses. In the telencephalon/ rostral preoptic area, TH mRNA-expressing cells were observed in different nuclei of the telencephalic hemispheres, and in the rostral preoptic area. However, steroid effects on TH expression as analyzed by ISH was observed only in a single nucleus located in the rostral preoptic area, the nucleus preopticus anteroventralis (NPOav): While TH labeling in this nucleus was low in control animals, a strong labeling was seen in androgen (T or DHT)-treated animals (Fig. 7, A and B). No difference in TH ISH labeling was observed after E2 treatment (data not shown). This is in complete accordance with the results from the qrtRT-PCR analyses.

Characterization of hypophysiotropic neurons by DiI retrograde tracing

DiI retrograde tracing from the pituitary was compared with data from ISH and immunohistochemistry to characterize potentially hypophysiotropic DA systems in the eel brain. No DiI staining was observed in the olfactory bulbs. In the anterior part of the preoptic area, strong DiI staining was observed in the NPOav, indicating direct hypophysiotropic connections from this preoptic nucleus (Fig. 8A). This nucleus also contains TH, as revealed both by immunohistochemistry (Fig. 8B) and ISH (Fig. 7). We also observed DiI staining in other hypothalamic nuclei, but as they were TH negative, they will not be included in this work.

Discussion

We have demonstrated that androgens exert regionally dependent, differential effects on the regulation of TH mRNA levels in the brain of prepubertal female European eel. Specifically, androgens (T or DHT) stimulated TH mRNA expression in the olfactory bulbs and in the nucleus preopticus anteroventralis (NPOav). In contrast, E2 had no effect in either area.

To our knowledge, this is the first report of androgen-dependent modulation of TH expression or activity in the olfactory bulbs in any vertebrate.

TH distribution in the eel brain

Using immunohistochemistry, Roberts et al (19) investigated the localization of DA itself in the eel brain. The results from (19) are in complete agreement with our results on TH localization by ISH in the fore- and midbrain (of which only a small part is shown in this article). The distribution of the other main catecholamine in the teleost brain, noradrenaline, has not been investigated in the eel, but results from other teleost species [e.g. the three-spined stickleback: Ekstrom et al (1990; J Chem Neuroanat 3:233), or zebrafish: Byrd et Brunjes (1995; J Comp Neurol 358:247)] all show that this neurotransmitter is synthesized only in the hindbrain, almost exclusively in the isthmic region (presumably equivalent to the locus coeruleus) and in the lateral parts of the nucleus of the solitary tract. Noradrenergic cell bodies are not found in the teleost fore- or midbrain (e.g. 18). In our experiments, a potential regulation of noradrenaline by steroids should be reflected on the TH expression in the eel brain part 'medulla oblongata'. However, neither qrtRT-PCR nor ISH analyses showed any effect of steroids in this brain region in our experiments. Because TH-positive cells in the teleost mid- and forebrain are DAergic, our results suggests an androgen-dependent increase in DAergic activity.

We determined TH mRNA levels in defined brain regions using a recently developed qrtRT-PCR assay (32). Highest relative levels were found in the olfactory bulbs, with 10 - 20 times higher expression levels compared to the diencephalic / mesencephalic areas and the telencephalon / rostral preoptic area. TH was detectable at low levels in the optic tectum and medulla oblongata, while no TH expression could be detected in the corpus cerebellum. As a qualitative support to the qrtRT-PCR analyses, and for further identification of steroid effects on specific TH-expressing nuclei, we investigated the cellular distribution of TH mRNA expression by ISH in brains taken from controls and steroid-treated females. The relative distribution of TH mRNA found in our experiments is in agreement with earlier preliminary results in the eel using Northern blot or RT-PCR (33), and also with immunohistochemical results from several teleosts, including TH (36) and DA distribution in the European eel (19). Moreover, our results on the relative TH brain distribution correspond well with those obtained in a similar analysis of female eel brains using the described qrtRT-PCR assay, underlining the accuracy and reproducibility of the brain dissection protocol and qrt-PCR analyses (32). The overall pattern of TH expression found in this work is also in agreement with that in higher vertebrates (38). However, although the relative brain distribution pattern may be similar between teleost species, and even within vertebrates in general, the absolute level of enzyme activity may vary. For example, Giorgi et al (39) reports DOPAC/DA ratios in the prepubertal European eel to be 4-15-fold higher than those in the early vitellogenic rainbow trout (*Oncorhynchus mykiss*) hypothalamus (40). Such differences may reflect species variations in brain development and function following adaptation to different life strategies.

Androgen-dependent stimulation of TH transcript levels in the olfactory bulbs

Our qrtRT-PCR analyses demonstrate an androgen (T or DHT) -dependent stimulation of TH mRNA levels in the olfactory bulbs. ISH analyses further localized the effect of androgens by showing increased expression throughout the periglomerular cell layer. Roberts et al (19) have previously shown these cells to be DAergic in the eel.

An androgen-dependent regulation of DA activity in the olfactory bulbs has important implications. The olfactory bulbs of most vertebrates are rich in intrinsic DA neurons in the periglomerular layer (41). In mammals, these cells, which show spontaneous firing of action potentials in a rhythmic fashion, modulate the activity of major output neurons in the olfactory bulbs and therefore are considered essential for olfactory processing (see 42, 43). DAergic modulation in the olfactory bulbs also seems involved in odor learning and memory (1, 2, 47, Pavlis et al 2006). Mechanistically, DA acts through D1-receptors to increase odor discrimination, while activation

of olfactory bulb D2-receptors results in decreased odor discrimination (Pavlis et al 2006) (1, 44 - 46).

Chemosensory cues are important factors governing social interactions and reproductive physiology in many species of vertebrates, and this is largely documented in mammals, including humans (see 48, 49). It is therefore not surprising to find that olfactory function is affected by gonadal factors. For instance, olfactory sensitivity in female rats is correlated with their estrous cycle with peak sensitivity during estrus (50). These cyclic changes are eliminated by castration, but can be restored by E2 treatment. Also, castration of male rats leads to a decreased response to female pheromones (51), suggesting that T increases pheromone sensitivity in males.

There are also numerous examples in the literature showing effects of androgens on DA or TH expression and activity in different vertebrate classes. However, to the best of our knowledge, there is no previous report of a strictly androgen-dependent effect on these systems. That is, where a non-aromatizable androgen (DHT in our study) produces the same effect as an aromatizable androgen (T in our study), and thus excluding the involvement of local aromatization of T in the brain tissue. The only exception is the increased TH immunoreactivity in the preoptic area in *male* frog brains after treatment with T or DHT (66).

Regarding whether TH or DA activity specifically in the vertebrate olfactory bulbs is affected by (sex) steroids, one previous report shows that E2 treatment decreased olfactory bulb TH mRNA levels in ovariectomized female mice (52). In teleosts, a previous report concluded that DA activity in the olfactory bulb is independent of gonadal development as there was no effect of E2 (androgens were not investigated) on ISH labeling of olfactory bulb TH cells from vitellogenic female rainbow trout (53).

Under natural conditions, plasma androgen levels in the European eel increase during silvering (transition from juvenile “yellow” stage to prepubertal “silver” stage) (24, 25), and are further increased during experimental maturation in both sexes (54). Giorgi et al (39) reported an increased DA activity in eel olfactory bulbs during silvering, and our results demonstrate that androgens stimulate DA activity in the olfactory bulbs. Together, this indicates that androgens may enhance central processing of olfactory cues, which have been suggested to be essential for navigation during the eel catadromous migration (23) towards the Sargasso Sea spawning grounds (55).

Androgen-dependent stimulation of TH transcript levels in the NPOav

We have demonstrated an androgen (T or DHT) -dependent stimulation of TH mRNA levels specifically in the NPOav of the rostral preoptic area. qrtRT-PCR analysis showed increased TH mRNA levels after androgen treatment in the telencephalon / rostral preoptic area, an effect which was not produced by E2. Furthermore, ISH analyses localized the increased TH mRNA expression specifically to the NPOav, a nucleus previously demonstrated to be DA positive in the eel (19). Our DiI tracing experiments showed the NPOav to be hypophysiotropic in the eel, as has also been shown in other teleosts (goldfish, *Carassius auratus*; 37). These DA cells in the NPOav are known in some adult teleosts to exert an inhibitory tone on the release of LH, thus controlling the final steps of gametogenesis (ovulation/spermiation) [goldfish (56); catfish, *Clarias gariepinus* (57); tilapia, *Oreochromis niloticus* (58)]. In the few species studied, E2 stimulates the DA (inhibitory) tone in the NPOav towards final maturation. In accordance with this, DA cells in the NPOav in trout express E2 receptors, providing a direct route for the increased DA tone upon E2 feedback (59). When tested in teleosts, T had the same effect as E2 (60) and was believed to act after local aromatization into E2, but the effect of non-aromatizable androgens has not been investigated.

In contrast to the DA inhibitory control of final maturation in teleosts, relatively few studies have addressed the DA inhibitory control of puberty. Where investigated, a non-existent involvement of DA in pubertal development was observed (61-63). In the eel, however, we have recently demonstrated that DA neurons originating in the NPOav exert a strong inhibition on pubertal development (22). The present study shows that in the prepubertal eel, androgens, and not E2 as in adults of other teleost species, increase DA activity in the NPOav, indicating that androgens may contribute to the early setting of DA inhibition of pubertal development in this species until the oceanic migration can take place. The difference in the steroid-specificity of DA regulation in the eel and other teleosts may reflect differences that are either species-specific or dependent on physiological stage.

Our previous work showed that T may have additional effects on the brain-pituitary-axis such as a direct stimulatory effect on pituitary LH beta expression (64, Huang et al., 1999), a synergistic effect with E2 on brain GnRH synthesis (20), and a possible sensitizing effect on pituitary LH response to GnRH (22). The seemingly paradoxical role of T in setting up the precocious DAergic inhibition, indicates a set of complex interactions. Our current hypothesis is that environmental factors may be involved in releasing the inhibitory effect of DA along the migratory route.

Androgen-dependent regulations and aromatase activity

In teleost fishes, T is generally believed to exert most of its effects upon local aromatization to E2. In contrast, our observed stimulation of TH mRNA in the female eel forebrain is clearly androgen-dependent, as treatment with T or DHT gave identical results in all areas, while E2 treatment has absolutely no effect in these areas. In line with these androgen-specific effects, earlier studies in the prepubertal female eel have shown differential effects of T and E2 on the gonadotropic axis, both in vivo and in vitro: on the brain and pituitary content of GnRH peptides (mGnRH and cGnRH-II) (20), and pituitary protein and transcript levels of gonadotropin subunits (20, 26, 64, 65). However, very few cases of purely androgen-dependent effects on brain DA systems have been reported in vertebrates (and none in female vertebrates): Chu and Wilczynski (66) demonstrated that androgen replacement (T or DHT) in gonadectomized male frogs increased the number of TH labeled cells in three main forebrain TH populations, the preoptic area, suprachiasmatic nucleus, and caudal hypothalamus/posterior tuberculum, but possible effects of estrogens were not investigated.

Different from the situation in mammals and other amniotes, androgens are synthesized in significant quantities in the gonads of both male and female fishes, including the eel in which E2 and androgens, both aromatizable (T) and non-aromatizable (5□-androstane-3□,17□-diol and 11-ketotestosterone) show similar titers in the female plasma (24, 25, 67). However, up to now, potential specific effects of androgens on brain DA systems have not been addressed, as the effect of T on the brain was believed to be exerted only after local aromatization into E2 (see 68). Accordingly, T had the same effect as E2 on brain DA turnover and LH release in goldfish (60). Moreover, teleosts generally have a much higher brain aromatase activity compared to mammals (69), with the preoptic area showing especially high transcript levels and activity (see 70). Two distinct aromatase genes have been revealed in some teleosts, one expressed essentially in the gonads and one essentially in the brain [goldfish (71); zebrafish, *Danio rerio* (72); tilapia (73); trout (74, 75)], providing a genetic basis for the high brain aromatase activity. However, in eels only one gene, most similar to the gonad-specific isoform, has been found (76, 77). Also, a recent study showed that brain aromatase enzyme-activity in eels is very low compared to other species (26, see also 27), thus paving the way for androgen-dependent regulations of the eel brain.

Obvious questions are then whether DAergic neurons express androgen receptors, and whether androgens exert a direct effect on TH expression. There is no information on the expression of androgen receptors in the teleost brain, but in the rainbow trout NPOav, about 60-70% of the TH-positive neurons also express E2 receptors (59), whereas all TH-positive neurons in the same nucleus also express glucocorticoid receptors (Teitsma et al 1999, Biol Rep 60:642-650). Accordingly, TH mRNA expression in the NPOav is stimulated by E2 in the trout (e.g. 15) and cortisol (e.g. Lewis et al 1987, PNAS 84:3550-3554) in various vertebrate classes.

Very recent results from Jeong et al (Neuroscience Letters, 2006, in press) show that, at least in mammalian cell lines, androgen receptors are able to transactivate TH promotor activity in a ligand-dependent manner, providing evidence for a direct effect by androgens on DAergic neurons.

These questions will be the focus of our future studies, both in relation to our own results, and also because of the recent characterization of two distinct androgen receptors in eel (Ikeuchi et al 1999, J Biol Chem 274:25205-25209; and Todo et al 1999, Biochem Biophys Res Comm 254:378-383).

Conclusion

Using qrtRT-PCR, ISH, immunohistochemistry, and pituitary retrograde DiI tracing, we have investigated the in vivo regulation of TH mRNA expression by gonadal steroids in a representative of an early-branching teleost group, the European eel. In prepubertal females, we have demonstrated an androgen-dependent positive feedback on DA neurons of the NPOav, which directly innervate the pituitary and inhibit gonadotropin release in teleosts. Moreover, we demonstrated an androgen-dependent stimulation of TH-immunopositive interneuron activity in the olfactory bulbs. This suggests an androgen-dependent enhancement of DA-regulated olfactory sensitivity and discrimination, and provides a new basis for the regulation by gonadal steroids of central DA systems in vertebrates.

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Figure captions

Figure 1. Sagittal scheme of a European eel brain showing the dissection of the different brain regions for the qrtRT-PCR analyses. Ob, olfactory bulbs; Tel/POA, telencephalon including rostral preoptic area; Ot, optic tectum (including dorsal tegmentum); Di/Mes, diencephalic and mesencephalic areas; Cb, corpus cerebellum; Mo, medulla oblongata.

Figure 1. Weltzien et al.

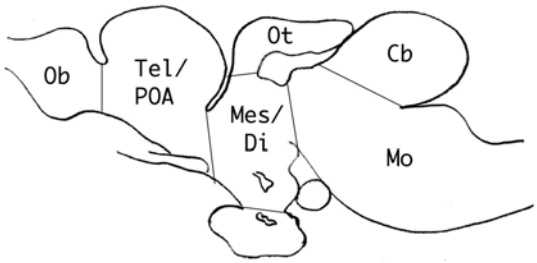

Figure 2. Transverse section showing DiI retrograde tracing from the eel pituitary. The inserted DiI microcrystal (arrowhead) is apparent in the ventromedial part of the proximal pars distalis (PPD) of the pituitary, while most of the fluorescent dye has diffused through the pars distalis and into the axonal endings (arrows, Ax) of the hypophysiotropic neurons, which directly innervate the adenohypophysis in teleosts. MBH: mediobasal hypothalamus; V3: third ventricle.

Figure 2. Weltzien et al.

Figure 3. Relative transcript levels of tyrosine hydroxylase (TH) in different brain regions of prepubertal female European eel, as quantified by qrtRT-PCR. Data are normalized to eel acidic ribosomal phosphoprotein P0 (ARP) and expressed as the fold difference from a total brain cDNA calibrator control. Ob, olfactory bulbs; Tel/POA, telencephalon including rostral preoptic area; Ot, optic tectum (including dorsal tegmentum); Di/Mes, diencephalic and mesencephalic areas; Cb, corpus cerebellum; Mo, medulla oblongata. Means are given ± S.E.M. (n = 6 – 8). Different letters indicate significant difference ($P < 0.05$; Kruskal-Wallis). nd, not detectable

Figure 3. Weltzien et al.

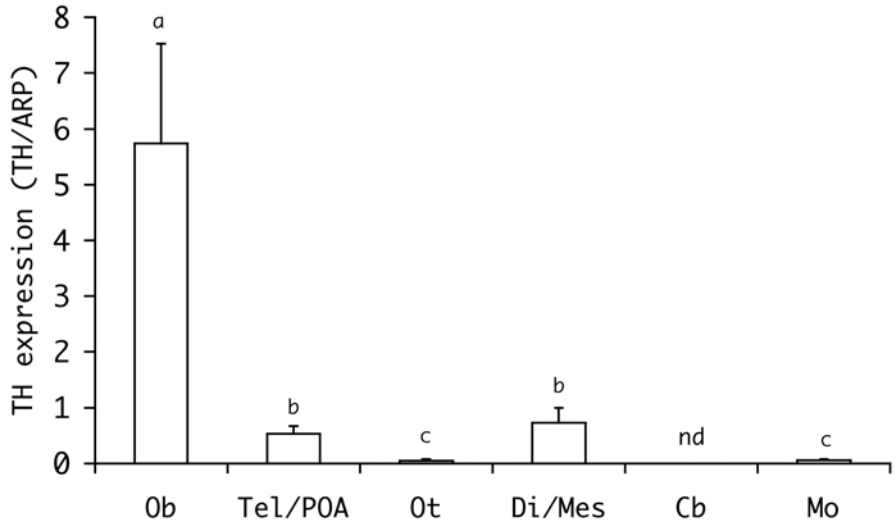

Figure 4. Effect of in vivo treatment with testosterone or estradiol-17 on TH transcript levels in different brain regions of the female silver European eel, as quantified by qrtRT-PCR (Experiment 1). Eels received eight weekly injections of steroid or saline (control). For normalization of data and for brain regions, see legend of Fig. 3. Means are given ± S.E.M. (n = 8). Significant differences between treated and control values from the same region: * $P < 0.05$; ** $P < 0.01$ (Kruskal-Wallis). nd, not detectable.

Figure 4. Weltzien et al.

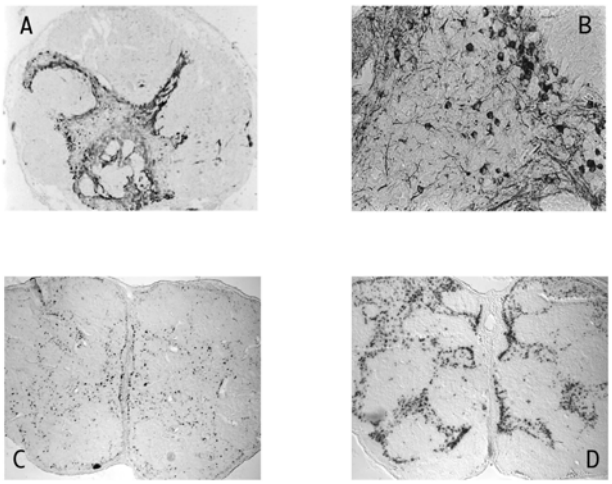

Figure 5. Effect of in vivo treatment with testosterone, estradiol-17□, or 5□-dihydrotestosterone (non-aromatizable androgen) on TH transcript levels in different brain regions of the female silver European eel, as quantified by qrtRT-PCR (Experiment 2). Eels received eight weekly injections of steroid or saline (control). For normalization of data and for brain regions, see legend of Fig. 3. Means are given ± S.E.M. (n = 8). Significant differences between treated and control values from the same region: * $P < 0.05$; ** $P < 0.01$ (Kruskal-Wallis). nd, not detectable.

Figure 5. Weltzien et al.

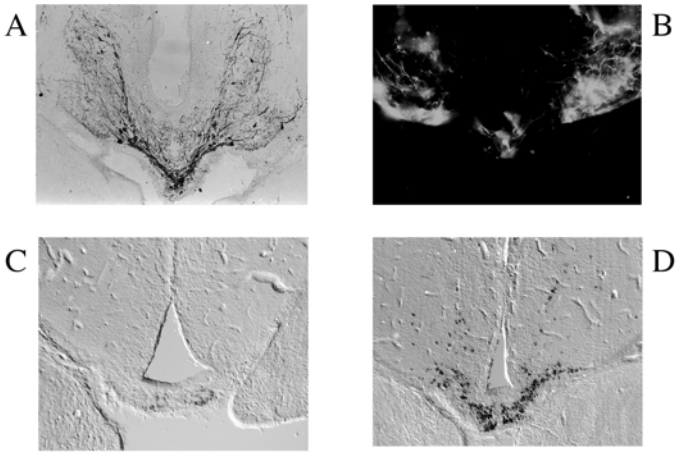

Figure 6. Transverse sections of olfactory bulbs from prepubertal female eels. In situ hybridization labeling of TH mRNA in control (**A**) and testosterone-treated (**B**) eels show increased TH mRNA in the periglomerular area upon testosterone treatment as compared to control. G, glomerulus; ECL, external cell layer. Scale bar 100 □m.

Figure 6. Weltzien et al.

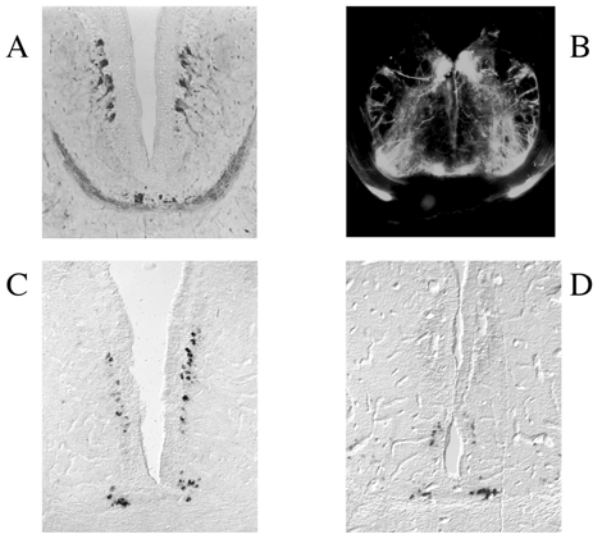

Figure 7. Transverse sections of the rostral preoptic area from prepubertal female eels. In situ hybridization labeling of TH mRNA in control (**A**) and testosterone-treated (**B**) eels show increased TH mRNA in the nucleus preopticus anteroventralis (NPOav) upon testosterone treatment as compared to control. V3, third ventricle; OT, optic tract. Scale bar 100 □m.

Figure 7. Weltzien et al.

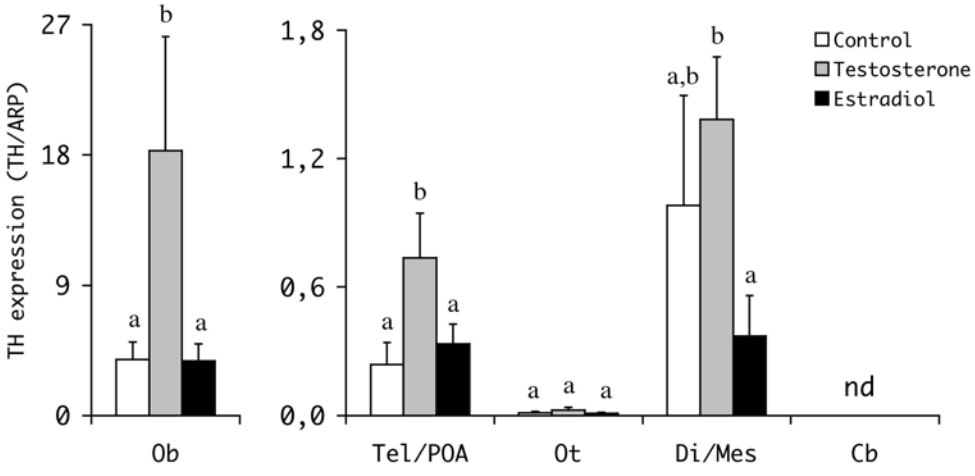

Figure 8. Transverse sections of the rostral preoptic area from prepubertal female eels. Cells in the NPOav are hypophysiotropic, as shown by the DiI retrograde tracing (**A**). TH immunohistochemical analyses (**B**) show specific staining of cell bodies in the nucleus preopticus anteroventralis (NPOav). V3, third ventricle; OT, optic tract. Scale bar 100 □m.

Figure 8. Weltzien et al.

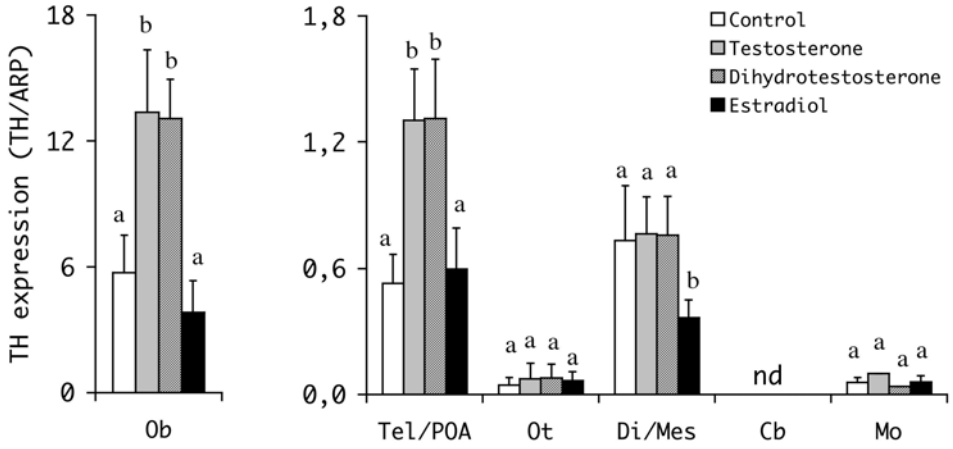

Table 1. Biometric parameters in control and steroid-treated female prepubertal European eels. Data are expressed as mean ± S.E.M. (n = 8). Eels received eight weekly injections of steroid (testosterone, 17□-estradiol, or 5□-dihydrotestosterone, DHT) or saline (control). BW, body weight; GSI, gonadosomatic index; HSI, hepatosomatic index were measured at the end of the experiment.

	Experiment 1			Experiment 2			
	Control	*Testosterone*	*Estradiol*	*Control*	*Testosterone*	*DHT*	*Estradiol*
BW (g)	249.6 ±38.0	320.1 ±126.6	329.4 ±108.5	350.4 ±52.7	355.0 ±44.8	311.6 ±51.4	398.0 ±70.3
GSI (%)	1.06 ±0.24	1.27 ±0.16	1.18 ±0.27	1.41 ±0.09	1.46 ±0.24	1.66 ±0.18	1.54 ±0.22
HSI (%)	1.18 ±0.16	1.12 ±0.13	1.40 ±0.22	1.16 ±0.19	1.14 ±0.15	1.21 ±0.21	1.26 ±0.11